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# STUDIES ON THE BIOCHEMISTRY OF TETRAHYMENA. IV. AMINO ACIDS AND THEIR RELATION TO THE BIOSYNTHESIS OF THIAMINE

GEORGE W. KIDDER AND VIRGINIA C. DEWEY [1]

*Arnold Biological Laboratory, Brown University, Providence, Rhode Island*

It was reported earlier (Kidder and Dewey, 1942) that two species of Tetrahymena were able to carry out the synthesis of thiamine, if provided with a substance found mainly in the leaves of plants. This substance was called Factor S and was found in highest concentration in alfalfa leaf meal but could not be demonstrated from materials of animal origin. Factor S was characterized by its solubility in water and alcohol (up to 75 per cent), insolubility in ether and acetone, stability to prolonged heat in the presence of either alkali or acid, and its stability to ultraviolet radiation. It was shown to be dialyzable through cellophane and not to be precipitated by the salts of heavy metals. It was shown that Tetrahymena gave optimal growth in a medium consisting of "vitamin-free" casein, salts and a heat- and alkali-treated water extract of alfalfa meal. Very little growth occurred in the absence of the alfalfa extract and the addition of thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, pimelic acid, *i*-inosital, uracil, or *p*-aminobenzoic acid either singly or in combination had no significant effect. Inasmuch as the heat- and alkali-treated alfalfa extract was certainly free of thiamine it was concluded that Tetrahymena could synthesize the thiamine required for its metabolic needs when supplied with Factor S. It was suggested that Factor S possibly acted as a catalyst necessary for the synthesis of the thiamine molecule.

It was recognized that the alfalfa extract used contained Factors I and II (Dewey, 1941; 1944) and we now know that the casein base contained Factor III (Kidder and Dewey, 1945a).

This work was criticized by Hall and Cosgrove (1944) on the basis that the "vitamin-free" casein used for the base medium was not free of thiamine. They reported growth of their strain of Tetrahymena in heat- and alkali-treated casein in the presence of thiamine and not in its absence. This criticism was shown to be invalid (Kidder and Dewey, 1944) when an extension of the earlier studies was carried out, using heat- and alkali-treated base media (casein, casein hydrolysate, gelatin, gelatin hydrolysate). It was then found that heat and alkali treatment of

[1] Aided by grants from the Morgan Edwards Fellowship Fund, the Manufacturers Research Fund for Bacteriology and Protozoology of Brown University.

whole casein produced toxic substances which could not be overcome by thiamine addition for *T. geleii* W but could to a slight extent for *T. geleii* H. In tryptophane-supplemented gelatin (Harris), however, indefinitely transplantable growth was possible after all of the thiamine had been destroyed. The addition of thiamine did not affect the generation time but did increase significantly the maximum yield and survival. The addition of heat- and alkali-treated alfalfa extract decreased the generation time and raised the maximum yield to optimal for the species, and the addition of thiamine had no significant effect. This was interpreted as meaning that gelatin possessed low concentrations of Factors I, II, and S and that the final cessation of growth was due to the depletion principally of Factor S, as the addition of thiamine did raise the maximum yield.

One of the difficulties encountered in the earlier work was the separation of Factor S from Factors I and II. The heat and alkali treatment of peptones seemed to destroy the Factor I activity, but toxic substances were produced which rendered the medium inferior for our tests. Nevertheless, it was possible to show that lead acetate precipitate (containing no factor S) from plant material could replace the heat- and alkali-destroyed fraction only if thiamine was added. This was taken to mean that peptone contained no Factor S but did contain Factor II which was stable to the treatment used for dethiaminization, and Factor I which was unstable. It was recognized that little more could be done until active preparations of Factors I II could be obtained which were essentially free of both Factor S and toxic materials.

Recently we have been able to obtain such a preparation and it has been possible to test the activity of Factor S. This work, to be reported here, while confirming our earlier conclusions on thiamine synthesis, has forced us to alter our original theory concerning the role of Factor S in the metabolic activities of Tetrahymena.

## Material and Methods

The organism used in the present study was the ciliated protozoan *Tetrahymena geleii* W, which is the strain used in the previous studies on thiamine synthesis (Kidder and Dewey, 1942; 1944). All work was done with pure (bacteria-free) cultures. The ciliates were grown in 2 ml. quantities of media in Pyrex tubes according to the technique described elsewhere (Kidder and Dewey, 1945b). All media, made with water twice distilled over permanganate in an all-Pyrex still, were adjusted to give a final pH of 6.8–7.0 and sterilization was by autoclaving. Serial transplants were made and results are recorded only after the third transplant. Transplants were made at 72 hour intervals using a bacteriological loop delivering approximately 0.008 ml. of fluid. Incubation was at 25° C. Population densities were determined by the direct counting technique (Kidder, 1941). All glassware used in this investigation was made chemically clean with sulfuric-dichromate solution, thoroughly rinsed and air dried before use.

In order to eliminate the possibility of cotton fibers contributing substances to the medium, Pyrex wool plugs were used extensively. It was found helpful to flame the protruding ends of the plugs until a thin crust had formed to eliminate the annoying strands inevitably present in this type of plug. This treatment fuses enough of the Pyrex strands to cause the plugs to hold their shape and increases appreciably the ease with which they may be handled.

Two types of base media were used for most of this work. One was 0.5 per cent hydrolyzed Eastman purified calfskin gelatin (Lot no. *144*). This hydrolysate was prepared by refluxing 100 gr. of gelatin in one liter of 25 per cent $H_2SO_4$ for 5 hours, removing the sulfate as $BaSO_4$ and reducing to the required concentration. Hydrolysate prepared with HCl was also used and the two were similar in every way. The gelatin hydrolysate was supplemented in all cases with 0.01 per cent *l*(−)-tryptophane and (with one exception to be noted later) with 0.02 per cent *dl*-valine. This base medium will be referred to as EGH.

The second type of base medium employed was a mixture of the eleven amino acids found to give optimum growth for this strain of *Tetrahymena geleii* (Kidder and Dewey, 1945a). These amino acids with the concentration in mg. per cent of each were as follows: *l*-(+)-arginine monohydrochloride—82; *l*(−)-histidine monohydrochloride—10; *dl*-isoleucine—35; *dl*-leucine—35; *dl*-lysine—60; *dl*-methionine—34; *dl*-phenylalanine—14; *dl*-serine—4; *dl*-threonine—20; *l*(−)-tryptophane—10; *dl*-valine—20. This base medium will be referred to as 11 AA. The sources of the amino acids used have been given elsewhere (Kidder and Dewey, 1945b).

Inasmuch as our primary concern was with thiamine all media were made up to contain other known growth factors, minerals and sugar to insure against limiting factors outside the scope of this investigation. Accordingly to our base media the following were always added:

| | mg./ml. |
|---|---|
| Difco bacto dextrose | 2.00 |
| $MgSO_4 \cdot 7H_2O$ | 0.10 |
| $K_2HPO_4$ | 0.10 |
| $CaCl_2 \cdot 2H_2O$ | 0.05 |
| $FeCl_3 \cdot 6H_2O$ | 0.00125 |
| $MnCl_2 \cdot 4H_2O$ | 0.00005 |
| $ZnCl_2$ | 0.00005 |

| | Micrograms/ml. |
|---|---|
| Biotin methyl ester | 0.00005 |
| Calcium pantothenate | 0.10 |
| Nicotinamide | 0.10 |
| *i*-Inositol | 1.00 |
| Choline chloride | 1.00 |
| *p*-Aminobenzoic acid | 0.10 |
| Pyridoxine hydrochoride | 0.10 |
| Uracil | 0.10 |
| Folic acid[2] | 0.01 |
| Riboflavin | 0.10 |

The sources of the salts and growth factors have been given earlier (Kidder and Dewey, 1945b).

Our preparation containing Factors I, II, and III was made from Liver Fraction L.[3] Fifteen grams of Liver Fraction L was dissolved in 750 ml. of distilled water, adjusted to pH 4.5, and extracted continuously for 96 hours in a liquid-liquid extracting apparatus (Wilson, Grauer, and Saier, 1940) with 750 ml. of *n*-butyl alcohol. The extracted material was freed of butyl alcohol, neutralized and

[2] Folic acid concentrate with a "potency" of 5000, furnished through the courtesy of Dr. R. J. Williams.

[3] Furnished through the courtesy of Dr. David Klein and the Wilson Laboratories.

the volume reduced to 300 ml. This was designated 12L, and was found to contain adequate amounts of Factors I, II, and III. The pH of this preparation was adjusted to 9.5–10.5 with NaOH and heated in the autoclave at 123° C. for one hour for dethiaminization. This preparation will be designated 12L1, which was found to be free of Factor S activity. 12L1 was used as a supplement in a final concentration of 1 : 20.

Preparations containing Factor S were obtained from alfalfa meal. Water extract of alfalfa, as previously described (Kidder and Dewey, 1942; 1944), was heated in the autoclave at 123° C. for one hour at pH 9.5–10.5 to insure the destruction of thiamine. This preparation, designated A, was used in a final concentration of 1 : 10.

## Results

When Liver Fraction L is heated with alkali to destroy thiamine, changes take place which make it inferior as a source of supplementary factors for Tetrahymena. The addition of thiamine does not completely overcome these toxic effects, although the inhibition is less than that produced when proteose-peptone is dethiaminized. It was found, however, that toxic materials were not produced upon heating provided the Liver Fraction L was extracted previously with butanol. The butanol extraction was used originally for the removal of pyridoxin and riboflavin (to be reported in detail later).

Table I

Growth in EGH and 11 AA with and without added Factor S from dethiaminized alfalfa extract (A) and with and without added thiamine. All tubes contain 12L1 and the numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

| Base | Additions | | | |
|---|---|---|---|---|
| | 0 | Thiamine | A | A + Thiamine |
| EGH | 3,100 | 305,000 | 75,000 | 290,000 |
| 11AA | 120,000 | 310,000 | 165,000 | 300,000 |

It was found that optimum growth resulted when a gelatin hydrolysate medium (with tryptophane, valine, and ten known growth factors), referred to as EGH, was supplied with 12L1 and thiamine, and very low growth occurred when the thiamine was omitted. The 12L1 was low in Factor S yet contained adequate amounts of Factors I, II, and III. This offered the opportunity to test the mode of action of Factor S, which could now be supplied from plant material without reference to the amounts of essential growth factors. Accordingly tests were set up using both EGH and 11 AA as base media, both supplemented with 12L1. To these base media were added various combinations of dethiaminized alfalfa extract (A) and thiamine. The results which were expected, namely the failure of growth unless either thiamine or Factor S was present, were not realized in 11 AA. Table I shows that very little growth occurred in the media based on EGH unless thiamine or Factor S was supplied but relatively good growth was obtained in the amino acid mixture in the absence of both. It will also be noted that thiamine is much more stimulatory, under these conditions, than is Factor S.

It was apparent from the foregoing results that the ability of Tetrahymena to synthesize thiamine was not dependent on the presence of Factor S when 11 AA was used as the base medium. This led to the conclusion that either some amino acid or combination of amino acids in the gelatin hydrolysate was blocking the synthetic mechanisms or that materials in the 12L1 were causing the block, the latter block being removed by some combination of the pure amino acids not present in the gelatin hydrolysate. The first of these possibilities was tested by making up an amino acid mixture based exactly on the published analysis for gelatin, but adding both tryptophane and valine (indispensable for this species). The ciliates behaved in this synthetic gelatin hydrolysate just as they had in 11 AA, so it was apparent that the first of the possibilities was untenable. The only known difference between the synthetic gelatin hydrolysate and EGH from a qualitative point of view was the inclusion in the former mixture of synthetic unnatural isomers (in the *dl* form, because of availability) of a number of the amino acids.

The addition of 11 AA to EGH plus 12L1 resulted in good growth without the addition of either thiamine or Factor S. This led us to test the effect of omitting each of the 11 amino acids singly from the 11 AA added to EGH. These results were inconclusive as fair growth occurred in all tubes. This was taken to mean that more than one of the 11 amino acids could counteract the inhibition to thiamine synthesis.

TABLE II

Growth of EGH with the addition of varying concentrations of racemic mixtures of amino acids. All tubes contain 12L1. The numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

| Amino acid | Concentration of amino acid added (mg./ml.) | | | | | |
|---|---|---|---|---|---|---|
| | 0.1 | 0.3 | 0.5 | 0.8 | 1.0 | Control, nothing added |
| dl-phenylalanine | 84,000 | 80,000 | 82,000 | 94,000 | 101,000 | 3,800 |
| dl-methionine | 92,000 | 8,600 | 8,000 | 6,000 | 7,800 | |
| dl-serine | 58,000 | 82,000 | 97,000 | 114,000 | 110,000 | |
| dl-norleucine | 31,000 | 11,500 | 0 | 0 | 0 | |
| dl-aspartic acid | 21,000 | 46,000 | 51,000 | 87,000 | 62,000 | |
| dl-isoleucine | 11,500 | 56,000 | 70,000 | 97,500 | 60,000 | |
| dl-lysine monohydrochloride | 4,500 | 15,000 | 58,000 | 61,000 | 78,000 | |
| dl-threonine | 6,000 | 31,000 | 42,000 | 66,000 | 81,000 | |
| dl-homcystine | 10,500 | 33,000 | 11,000 | 8,000 | 6,400 | |
| dl-alanine | 3,000 | 4,200 | 26,000 | 11,000 | 4,500 | |
| dl-glutamic acid | 6,500 | 7,500 | 12,500 | 21,000 | 37,000 | |

The next set of experiments was designed to determine whether or not the addition of single amino acids to the gelatin hydrolysate medium could counteract the inhibition to thiamine synthesis. Arbitrary amounts of each of nineteen amino acids were added to EGH. Thiamine synthesis occurred to a marked degree in some of the tubes, moderately in others and very little in some. In all cases where the inhibition was not removed the amino acid used was in its natural form while those amino acids which were most effective were synthetic.

This set of experiments was repeated using varying concentrations of the synthetic amino acids and some of the results are given in Table II. It will be seen that the effectiveness of the amino acids in releasing the inhibition of thiamine synthesis varied with the amino acid and the concentration. Phenylalanine was the most effective throughout the range of concentrations used while methionine was most effective in the lowest concentration. Norleucine was moderately effective at a concentration of 0.1 mg. per ml. but was toxic at 0.5 mg. per ml. or higher. These results indicated that the unnatural isomers were in some way able to release the inhibition of thiamine synthesis. It seemed more probable that the ratio between the two isomers was not the explanation, as some release of inhibition was found with some of the nonsynthetic amino acids. It is known that in the preparation of amino acids from natural sources some racemization is likely to occur and this might account for the small amount of activity.

TABLE III

Comparison of the effect of the natural isomer (1+) and the unnatural isomer (1−) of isoleucine, added to EGH + 12L1. Numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

| Amino acid | Concentration of amino acid added (mg./ml.) | | | | | |
|---|---|---|---|---|---|---|
| | 0.1 | 0.3 | 0.5 | 0.8 | 1.0 | Control, nothing added |
| 1(+)-isoleucine | 7,800 | 4,100 | 6,300 | 9,800 | 11,500 | 3,100 |
| 1(−)-isoleucine | 42,000 | 91,000 | 81,000 | 68,000 | 21,000 | |

This was shown to be the probable explanation by two sets of experiments. We had samples of natural *l*(+)-isoleucine, unnatural *l*(−)-isoleucine and synthetic *dl*-isoleucine. A comparison of the figures for *dl*-isoleucine in Table II with those in Table III shows that *l*(−)-isoleucine is effective in approximately one half the required concentration of *dl*-isoleucine. This is what is to be expected if only the unnatural isomer is effective in the removal of thiamine synthesis inhibition. The effectiveness of *l*(+)-isoleucine is low and increases with the concentration. This could be due to the occurrence of some racemization during its preparation.

TABLE IV

Comparison of the effect of the natural isomer (1−) and the racemic mixture (dl) leucine, added to EGH + 12L1. Numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

| | Concentration of amino acid added (mg./ml.) | | | | | |
|---|---|---|---|---|---|---|
| | 0.1 | 0.3 | 0.5 | 0.8 | 1.0 | Control, nothing added |
| 1(−)-leucine | 2,500 | 3,500 | 10,500 | 14,000 | 13,500 | 3,800 |
| dl-leucine | 4,100 | 5,500 | 26,000 | 29,000 | 31,000 | |

When natural leucine was compared to *dl*-leucine the former was found to be less effective in the release of the synthesis inhibition (Table IV). The difference

here, however, was not as marked, as the natural form appears to contain a considerable quantity of racemic mixture and the synthetic leucine is rather low in activity. It should be noted that we used Kahlbaum *dl*-leucine as this was found previously (Kidder and Dewey, 1945b) to be free of isoleucine, a common contaminant of many brands of synthetic leucine (Hegsted and Wardwell, 1944).

Inasmuch as EGH contained added *dl*-valine it was thought advisable to determine whether the unnatural isomer of this amino acid might be responsible for the ability of the ciliates to grow at all without added thiamine, Factor S or unnatural isomers of amino acids (see controls in Tables I–IV). Accordingly EGH minus valine was tested with varying concentrations of *dl*-valine with and without thiamine. Table V shows that without thiamine, very little growth occurs with no

TABLE V

Effect of the addition of dl-valine to EGH (minus valine). All tubes contain 12L1. Numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

| | Concentration of dl-valine (mg./ml.) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 0.05 | 0.1 | 0.3 | 0.5 | 0.8 | 1.0 |
| Minus thiamine | 150 | 2,500 | 4,000 | 7,500 | 16,000 | 37,000 | 45,000 |
| Plus thiamine | 190,000 | 210,000 | 265,000 | 310,000 | 305,000 | 325,000 | 315,000 |

added valine, and that the inhibition to thiamine synthesis is counteracted more effectively the higher the concentration of added *dl*-valine. With added thiamine, however, the addition of valine had little effect. This indicates that the sample of gelatin used differs from our previous sample of Eastman de-ashed gelatin in that it contains nearly optimum amounts of natural valine for this species. It had previously been found (Kidder and Dewey, 1945b) that Eastman de-ashed gelatin would not support growth of *Tetrahymena gelcii* W without added valine, even in the presence of thiamine. The fact that transplantable, though very low, growth occurs without the addition of any unnatural isomers of amino acids may mean that the inhibition to thiamine synthesis is never complete or that some racemization of the amino acids has occurred during hydrolysis.

When thiamine was added (0.1 micrograms per ml.) to any of the above described combinations, growth was always raised to approximately 300,000 ciliates per ml. Thiamine, therefore, although it can be synthesized by the ciliates, is very active as a stimulatory substance. It was of interest and importance to determine the amount of stimulation produced by different concentrations of thiamine when added to EGH plus 12L1; EGH plus 12L1 and one of the active amino acids; EGH plus 12L1 and Factor S; and 11 AA plus 12L1. Figures 1–4 show a summary of the activity of various concentrations of thiamine. The lowest concentration tested was 0.005 millimicrograms per ml. and in every case this amount gave significant stimulation. The stimulation was roughly proportionate to the concentration up to 0.001 micrograms per ml. In all cases, after this point, the amount of growth was increased more gradually but reached approximately the 300,000 level at 0.01 micrograms per ml. of thiamine when inhibition to thiamine synthesis was absent or removed. Ten times this amount of thiamine was required to raise

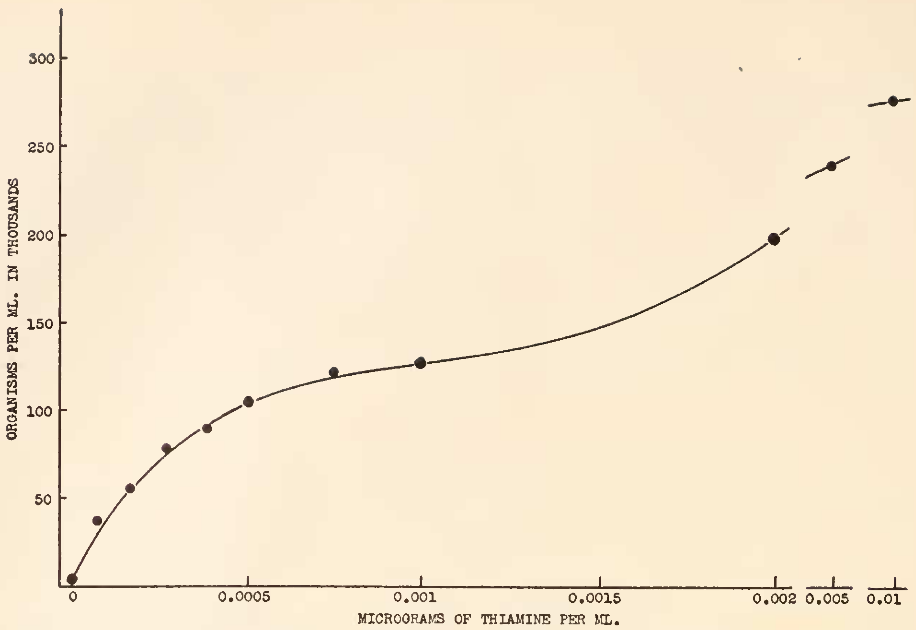


FIGURE 1. Curve of population densities at various concentrations of thiamine hydrocloride with gelatin hydrolysate (EGH) and dethiaminized butanol extracted Liver Fraction L (12L1) as base. The concentration of organisms was determined from the third transplant after 72 hrs. of growth.

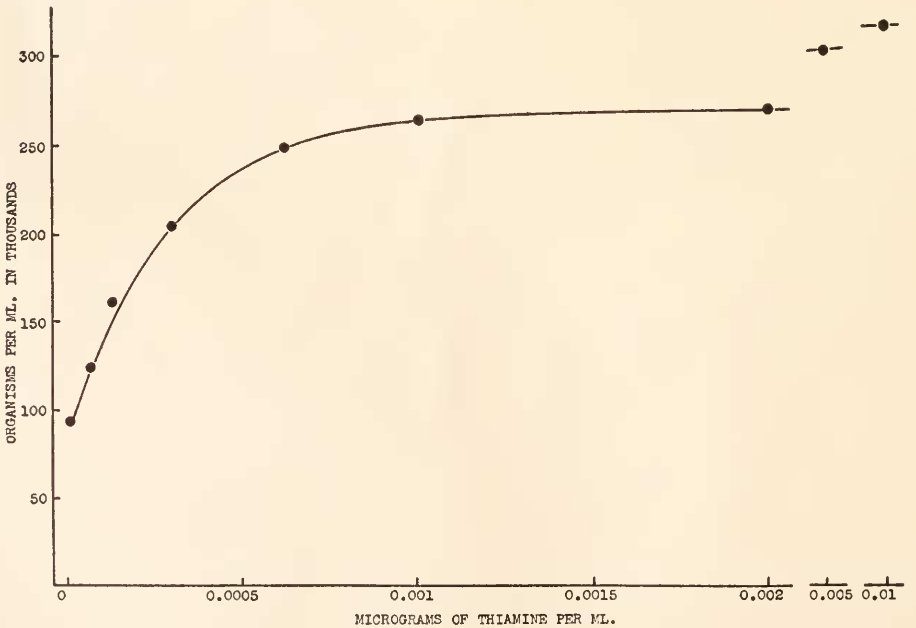


FIGURE 2. Curve of population densities at various concentrations of thiamine hydrochloride with EGH, 12L1 and *dl*-serine (0.5 mg./ml.) as base. Third transplant determinations after 72 hrs. of growth.

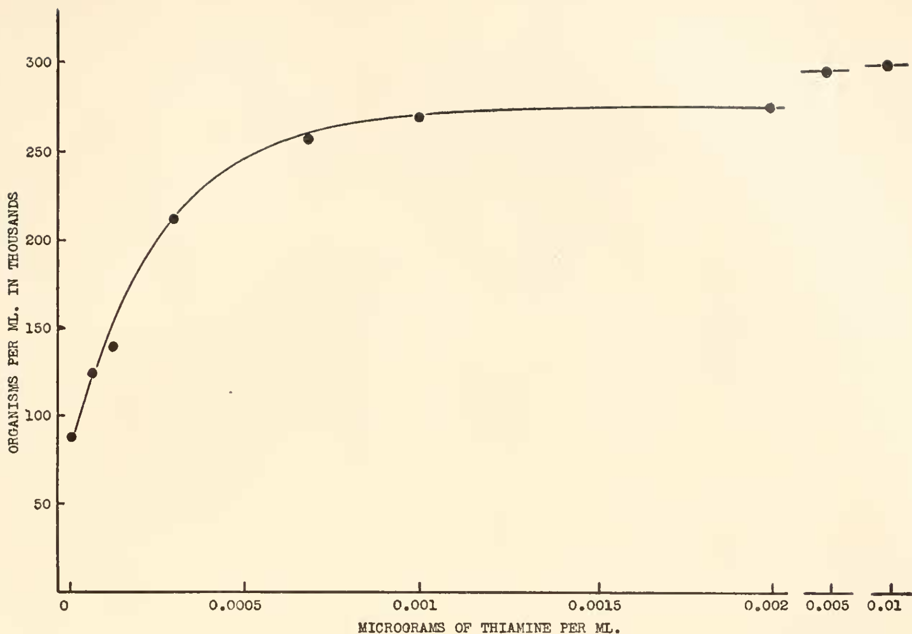


FIGURE 3. Curve of population densities at various concentrations of thiamine hydrochloride with EGH, 12L1 and dethiaminized alfalfa extract (A) as base. Third transplant determinations after 72 hrs. of growth.

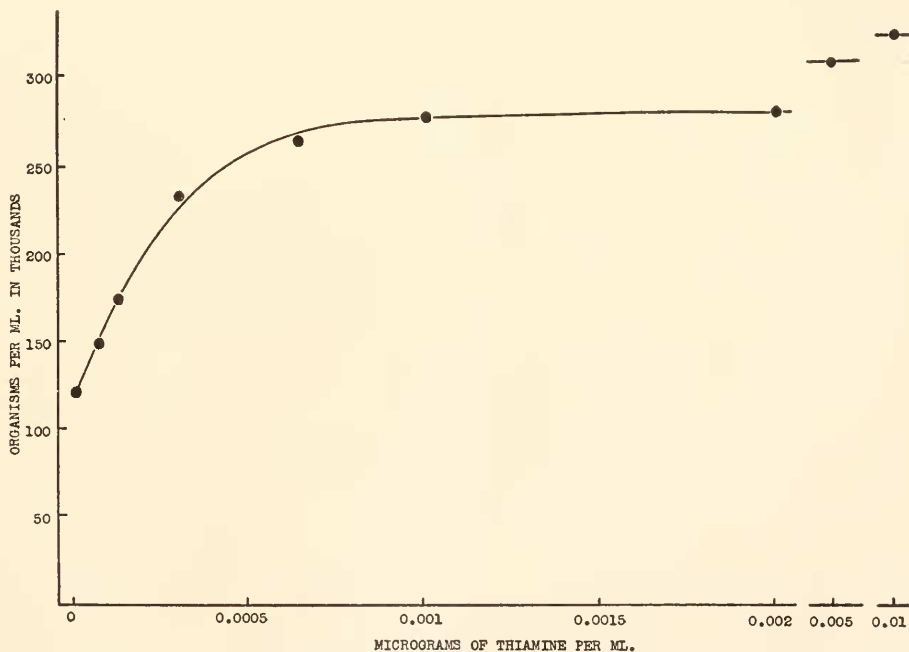


FIGURE 4. Curve of population densities at various concentrations of thiamine hydrochloride with the amino acid mixture (11 AA) and 12L1 as base. Third transplant determinations after 72 hrs. of growth.

the population to 300,000 per ml. where inhibition was pronounced (Fig. 1). These results show that Tetrahymena is far more sensitive to thiamine below a concentration of 0.001 micrograms per ml. than to higher concentrations.

An interesting and perhaps important point to be noted in the data shown in Figure 1 is the inflection which occurs in the curve above the 0.001 microgram per ml. level. The reasons for this inflection are not clear, although it seems possible that thiamine may be performing a double role where inhibition is pronounced. It may be supplying the vitamin needs of the organisms at the lower levels and acting to remove other inhibitions to growth as the concentrations increase.

Only the intact molecule of thiamine is capable of giving optimum stimulation. When the pyrimidine portion of thiamine (2-methyl-5-ethoxymethyl-6-amino pyrimidine) [4] or the thiazole portion (4-methyl-5-beta-hydroxyethyl thiazole) [4] were added separately or together some release of inhibition occurred. Table VI shows

TABLE VI

Growth in EGH plus 12L1 with varying concentrations of the thiazole and pyrimidine components of the thiamine molecule. Numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

| | Concentration (micrograms/ml.) | | | | | |
|---|---|---|---|---|---|---|
| | 0.001 | 0.005 | 0.01 | 0.1 | 0.5 | Control, nothing added |
| Thiazole | 3,000 | 8,400 | 20,000 | 1,400 | 1,000 | 2,900 |
| Pyrimidine | 1,200 | 5,300 | 17,500 | 3,200 | 2,600 | |
| Thiazole and pyrimidine (total conc.) | 2,500 | 11,000 | 24,000 | 2,400 | 2,500 | |

the results of these experiments. Both thiazole and pyrimidine produce stimulation in low concentrations but are mildly toxic at concentrations of 0.1 micrograms per ml. or higher. Thiazole and pyrimidine behave much the same as Factor S or the unnatural isomers of the amino acids, although to a less degree. They appear to cause the release of the thiamine synthesis inhibition and are themselves inhibitory in high concentrations.

## DISCUSSION

It appears from the foregoing results that there are substances present in natural materials which can block the synthetic mechanisms of Tetrahymena. Under the conditions of our experiments this blocking occurred specifically in the mechanism or mechanisms for the synthesis of the thiamine molecule. That this ciliate can synthesize thiamine, as was pointed out earlier (Kidder and Dewey, 1942; 1944) cannot be doubted, when the blocking substance is absent or the block is released. In our earlier work (Kidder and Dewey, 1942; 1944) where dethiaminized alfalfa extract was used as the supply of Factors I and II (Factor III was present in the casein and gelatin preparations; Kidder and Dewey, 1945a), it might be questioned whether the growth obtained in the absence of thiamine might be the result of no inhibitory substance rather than the presence of Factor S. However, it must be re-

[4] Both the thiazole and the pyrimidine used were furnished through the courtesy of Dr. George W. Lewis and Merck and Co.

membered that the addition of alfalfa extract to EGH plus 12L1 (which contains the inhibitory substance) removed the block. Whatever Factor S is, it is able to release the block to thiamine synthesis. But it is also seen that the unnatural isomers of the amino acids can act in a similar manner, so this reaction is far from specific as to counteracting substances. It was formerly proposed (Kidder and Dewey, 1942) that Factor S might act as a catalyst to the reaction wherein the thiamine molecule was synthesized. This hypothesis appears to be no longer tenable.

It does not seem likely that Factor S is, in reality, nothing more than racemic amino acids, for two reasons. If enough racemization occurred during the heat treatment of the alfalfa extract to account for the activity found then the same amount of racemization should have taken place in the heat treatment of 12L to produce 12L1. It was found, moreover, upon assaying the alfalfa extract for the indispensable amino acids for Tetrahymena that it did not contain enough of any one of the ten to support growth, when used in the concentration employed here. But a similar assay of 12L1 demonstrated almost optimum amounts of lysine; approximately half optimal amounts of arginine, threonine, and valine; and traces of histidine, isoleucine, leucine, and phenylalanine. It seems at present that Factor S represents some material present in alfalfa and the leaves of other plants (Kidder and Dewey, 1942), the activity of which is shared by the unnatural isomers of many of the amino acids.

The relation of amino acids to the ability of organisms to synthesize vitamins has been pointed out before. Snell and Guirard (1943) showed that alanine could replace pyridoxine for *Streptococcus fecalis* R (*S. lactis* R) and that alanine functioned to counteract the toxicity of glycine. It does seem strange, however, that the unnatural isomers appear to function in the release of thiamine synthesis inhibition for Tetrahymena. In nature this organism, being largely a bacteria feeder, probably would never be called upon to use its thiamine synthesis mechanism. The use of its ability to synthesize thiamine, therefore, is admittedly the result of artificial environmental conditions, as is also the very contact with the unnatural isomers of the amino acids.

It is apparent that, although Tetrahymena does possess the ability to synthesize thiamine, this vitamin is a potent stimulant to reproduction, size (Kidder and Dewey, 1944), and longevity (Johnson and Baker, 1943). Thiamine must, therefore, be included in complete media for this ciliate, but the amount needed appears to be less than has been previously used (Hall and Cosgrove, 1944; Kidder and Dewey, 1942; 1944).

It has been stated previously (Lwoff and Lwoff, 1938; Kidder and Dewey, 1942; 1944; Hall and Cosgrove, 1944) that heating peptones or proteins with alkali renders the media inferior for the growth of Tetrahymena. This condition could be partially counteracted for some strains by the addition of thiamine. The explanation appears now to rest in the partial destruction of serine, for we have found that if 11 AA is heat- and alkali-treated growth (with added 12L1) is very low but returns to normal with the addition of serine. Increased growth results with the addition of thiamine alone, however, indicating that this vitamin can replace serine. Or that serine (a dispensable but highly stimulatory amino acid in the presence of thiamine; Kidder and Dewey, 1945b), is one of the necessary factors for the synthesis of vitamin $B_1$.

The relationship which exists between the concentration of thiamine and the concentration of ciliates (Figures 1–4) might suggest that this organism would be useful for assay purposes. It would be difficult, however, to assay natural products for thiamine in a base medium composed of EGH plus 12L1, the only combination which gives a low blank, because of the likelihood of the introduction of Factor S or other materials of like nature with the substance to be assayed. Although we have not attempted to do this, it might be possible to arrange conditions so that 11 AA (Fig. 4) could be used and the values calculated as differences. Experiments directed to this end might prove valuable as the present microbiological methods are not entirely satisfactory. The majority of organisms used are stimulated by the thiamine components as well as by the whole molecule (Sarett and Cheldelin, 1944), require complex base media (Williams, 1942), or require many days of growth before results can be obtained (Robbins and Kavanagh, 1937).

## Summary

1. In Eastman gelatin hydrolysate (EGH) and Factors I, II, and III from Liver Fraction L (heat- and alkali-treated to destroy thiamine) the ciliate *Tetrahymena geleii* W grows very poorly without added thiamine.

2. A mixture of amino acids (11 AA) with the dethiaminized liver fraction supports fair growth without added thiamine.

3. There appear to be substances in the liver fraction or the gelatin hydrolysate or both which specifically block the mechanism for the biosynthesis of thiamine.

4. This block can be released by Factor S from alfalfa extract or by the unnatural isomers of a number of amino acids.

5. Some release of the inhibition to thiamine synthesis is produced by a few of the natural amino acids but this is probably due to the presence of low concentrations of unnatural isomers which result from racemization during preparation.

6. The unnatural isomer of isoleucine (the only unnatural isomer available for testing) was found to be active in approximately one half the concentration of the *dl*-isoleucine.

7. Thiamine is extremely stimulatory in low concentrations.

8. The thiazole and pyrimidine components are slightly stimulatory but this stimulation appears to be due to their ability to cause some release of the thiamine synthesis inhibition.

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